

TARGETING THE PROTEIN TYROSINE PHOSPHATASE, SHP2,
AND PI3K IN FLT3-ITD+ LEUKEMIA

Joshua D. Bowling

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Master's Thesis Committee

Rebecca Chan, MD, PhD, Chair

Sophie Paczesny, MD, PhD

Michael Ferguson, MD, MS

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Joshua D. Bowling

TARGETING THE PROTEIN TYROSINE PHOSPHATASE, SHP2, AND PI3K IN FLT-3
ITD+ LEUKEMIA

Internal tandem duplications in the *fms*-like tyrosine kinase receptor (FLT3-ITDs) cause constitutive activation of the receptor and confer a poor prognosis in acute myeloid leukemia (AML). We hypothesized that Shp2 interacts with FLT3-ITD via protein complexes at tyrosine (Y) 768, 955, and/or 969 and that Shp2 and PI3K work cooperatively to promote FLT3-ITD-induced leukemogenesis. Consistently, mutation of N51-FLT3 tyrosine 768 to phenylalanine reduced proliferation and levels of phospho-Erk compared to N51-FLT3-expressing cells while having no effect on levels of phospho-STAT5. In transplants, C3H/HeJ mice injected with either WT-FLT3-, N51-FLT3-, or N51-Y768F-expressing cells showed that mutation of Y768 had no effect on overall survival. In addition, pharmacologic inhibition of Shp2 with II-B08 or PI3K with GDC-0941 in N51-FLT3-expressing cells and primary patient samples showed decreased proliferation. A possible mechanistic explanation for reduced proliferation and selective reduction of P-Erk levels in the N51-FLT3-Y768-expressing cells is through decreased recruitment of Grb2, which participates with son of sevenless, SOS, to activate the RAS-Erk signaling pathway. The lack of improvement in overall survival could be due to preserved STAT5 signaling, as observed during in vitro experiments. Collectively, these data suggest that the tyrosine 768 residue plays an important role in phospho-Erk signaling in N51-FLT3-expressing cells, and that pharmacologic therapy with Shp2 or PI3K inhibitors may provide a novel treatment approach for FLT3-ITD positive AML. For future directions, we plan to treat mice with the Shp2 inhibitor, II-B08, the PI3K inhibitor, GDC-0941, or a combination to determine the effect on overall survival.

Rebecca Chan, MD, PhD, Chair

TABLE OF CONTENTS

CHAPTER ONE INTRODUCTION.....	1
SUMMARY.....	9
SIGNIFICANCE.....	10
CHAPTER TWO MATERIALS AND METHODS.....	11
RESULTS.....	16
CHAPTER THREE DISCUSSION	26
REFERENCES	28
CURRICULUM VITAE	

CHAPTER ONE INTRODUCTION

Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a heterogeneous disease characterized by clonal proliferation of myeloid precursor cells, with a reduced ability to differentiate into mature cells. It accounts for roughly 25% of all adult leukemias, with the American Cancer society estimating that 19,950 people will be diagnosed with AML in the US during 2016 (Deschler and Lubbert 2006; ACS 2016). Although this disease affects both the young and the elderly, the majority of patients are over the age of 65 (Deschler and Lubbert 2006)(Figure 1.1). Progress has been made in improving survival in younger populations – the 5-year survival in patients aged 15-24 in England has improved from 7% to 53% during 1971-2006; however, in patients aged 60-69 the improvement has been more modest, from 2% to 13% during this same time period (Shah 2013)(Figure 1.2). With an aging population, the numbers of affected individuals are projected to increase, creating an urgent need for improved treatments and curative therapy for AML. However, the increased age of patients also brings increased medical co-morbidities and reduced tolerance of cytotoxic chemotherapy and radiation (Deschler and Lubbert 2006; Estey and Dohner 2006; Melchert 2006). Therefore, novel therapies with reduced toxicity are needed to treat patients with AML who would otherwise receive only supportive care.

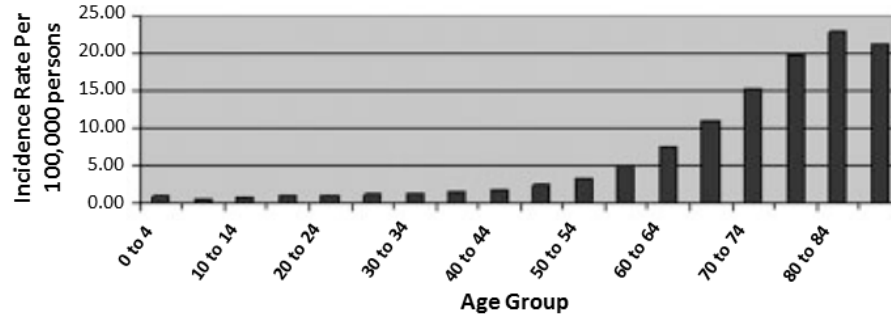


Figure 1.1: AML predominately affects those over the age of 60 years.

Graphical representation of the number of people diagnosed with AML out of 100,000, adapted from Deschler and Lubbert (2006).

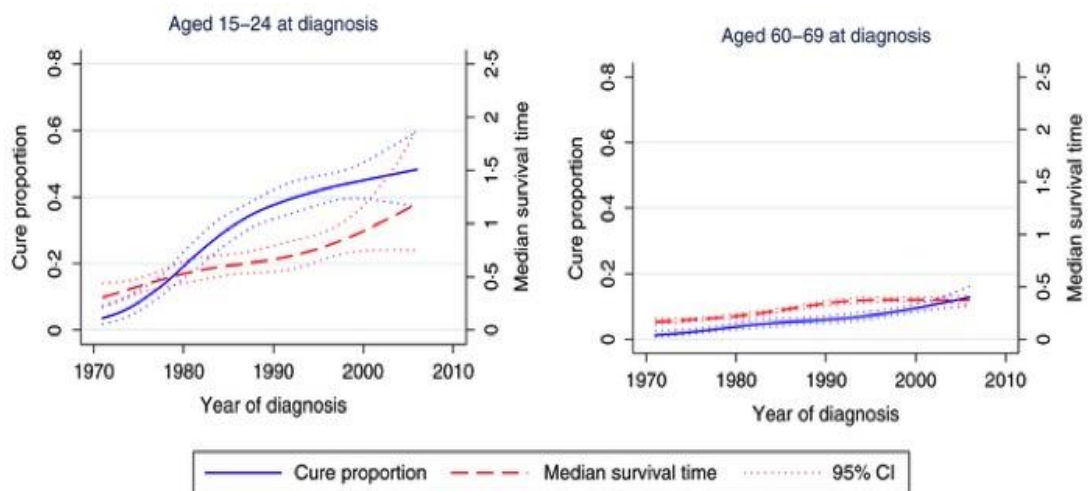


Figure 1.2: Proportion of patients cured of AML by year of diagnosis.

Graphical representation showing the proportion of patients achieving cure in each age group, arranged by year of diagnosis from 1971-2006, adapted from Shah, et al. (2013).

FLT3-ITD mutations in AML

Internal tandem duplications in the FMS-like tyrosine kinase (FLT3-ITD) are insertions of amino acids near the juxtamembrane domain and are present in approximately 25% of all AML patients, conferring a poor prognosis (Nakao, 1996; Thiede, 2002; Kottaridis, 2001). An illustration of WT-FLT3 and several described ITD insertions is presented in Figure 1.3 (Choudhary, 2005; Mizuki, 2000; Kelley, 2002). The importance of FLT3-ITDs as a poor prognostic factor in AML is established, and has been demonstrated by numerous investigators (Abu-Duhier, 2000; Kottaridis, 2001, Schlenk, 2008). The decreased survival of patients with FLT3-ITD+ AML compared to FLT3-ITD negative AML is illustrated in Figure 1.4.

FLT3-ITD mutations result in constitutive FLT3 phosphorylation and FLT3 ligand (FL) independent hyperproliferation (Mizuki, 2003; Murata, 2003; Hayakawa, 2000; Kiyoi, 2002). FLT3-ITDs also cause a promiscuous activation of signal transducer and activator of transcription 5, STAT5, which is known to activate anti-apoptotic proteins such as Bcl-X_L (Spiekermann, 2003; Hayakawa, 2000; Choudhary, 2007; Rocnik, 2006). Previous work in our lab has shown the importance of Shp2, a protein tyrosine phosphatase, in contributing to the leukemic phenotype in FLT3-ITDs (Nabinger, 2013). However, with Shp2 inhibition there was not a complete correction of the hyperproliferation seen in FLT3-ITDs, which has led to this current study. We wish to investigate the role of phosphatidylinositol-3-kinase (PI3K) and its cooperative role with Shp2 in FLT3-ITD-induced leukemia.

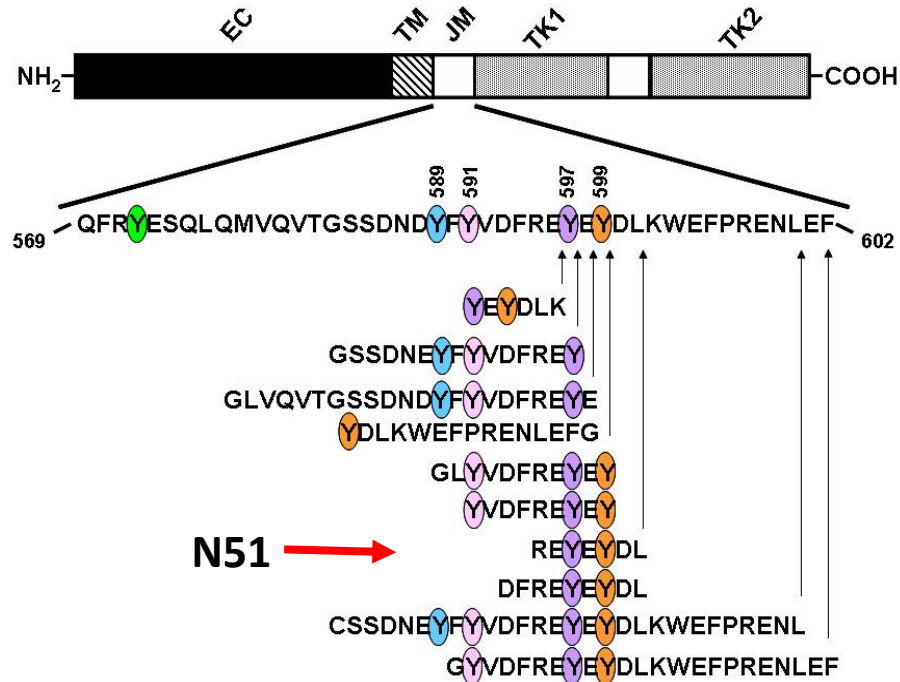


Figure 1.3: Representative schematic diagram showing duplication in ITDs.

Schematic diagram of several internal tandem duplications that have been observed in AML, including the N51-FLT3 used for the experiments in this project (Choudhary, 2005; Mizuki, 2000; Kelley, 2002). EC – extracellular, TM – transmembrane, JM-juxtamembrane, TK-1 – kinase domain, TK-2 – kinase domain.

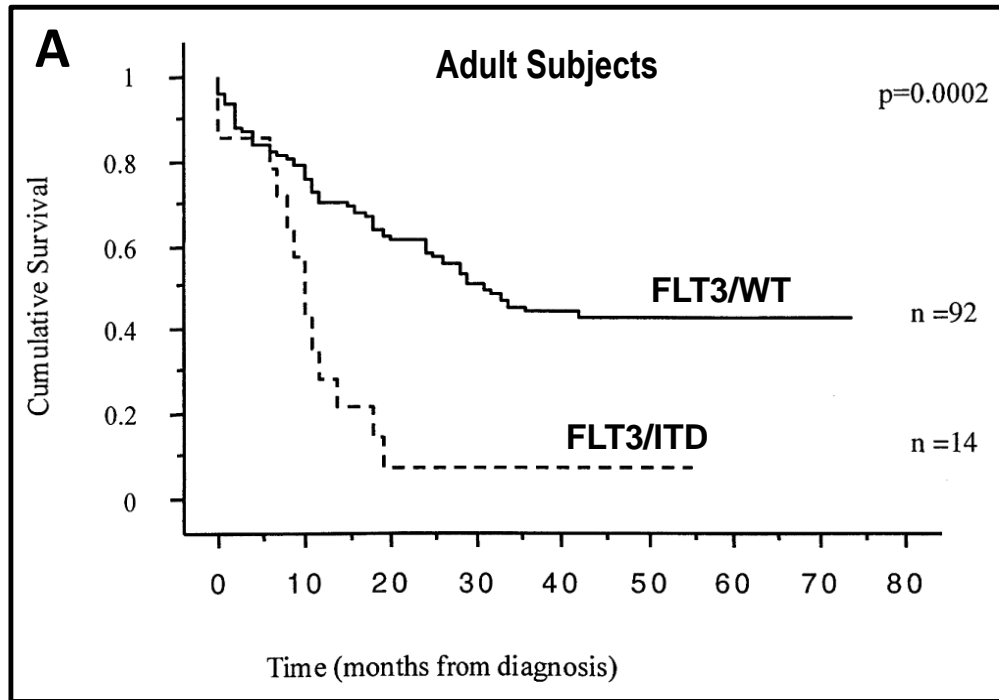


Figure 1.4: Cumulative survival of FLT3-ITD+ vs. FLT3-ITD- patients.

Kaplan-Meier curve showing cumulative survival of AML patients with or without the presence of a FLT3-ITD mutation. Adapted from Abu-Duhier, et al. (2000).

Role of the protein tyrosine phosphatase, Shp2, in FLT3-ITD+ AML

Shp2 is a protein tyrosine phosphatase that is ubiquitously expressed and critical for normal hematopoiesis (Qu, 1998). Furthermore, there is evidence of gain of function Shp2 mutations in hematologic malignancies, as well as overexpression of Shp2 in human AML samples (Tartaglia, 2003; Loh, 2004; Bentires-Alj, 2004; Xu, 2005). There is also a known interaction of Shp2 with WT-FLT3 at the tyrosine 599 residue, leading to cellular proliferation through the mitogen activated protein kinase (MAPK) pathway (Heiss, 2006). Given the role of Shp2 in normal hematopoietic function, and its presence in hematologic malignancies, the hypothesis that it contributes to the leukemic phenotype in FLT3-ITDs was explored by previous work in the lab (Nabinger, 2013). We found that Shp2 was constitutively associated with the FLT3-ITD, N51-FLT3, as well as with STAT5. In addition, we found that pharmacologic inhibition of Shp2 with a novel inhibitor, II-B08, reduced the proliferation of N51-FLT3 expressing cells and primary AML samples (Nabinger, 2013). However, co-immunoprecipitation (Co-IP) showed that STAT5 and phospho-STAT5 were still associated with FLT3-ITD following knock-down of Shp2 (Figure 1.5A), and thymidine incorporation assays revealed reduced, but not normalized, levels of cellular proliferation following genetic knockout of Shp2 in N51-FLT3-expressing cells (Figure 1.5B). This finding implies that, while Shp2 contributes towards STAT5 activation and cellular hyperproliferation in N51-FLT3-expressing cells, its loss does not completely normalize the leukemic phenotype. This led to our current study, which examines if the lipid kinase, phospho-inositol-3-kinase (PI3K) is playing a role in FLT3-ITD-induced leukemia and to what degree it may be having a cooperative effect with Shp2.

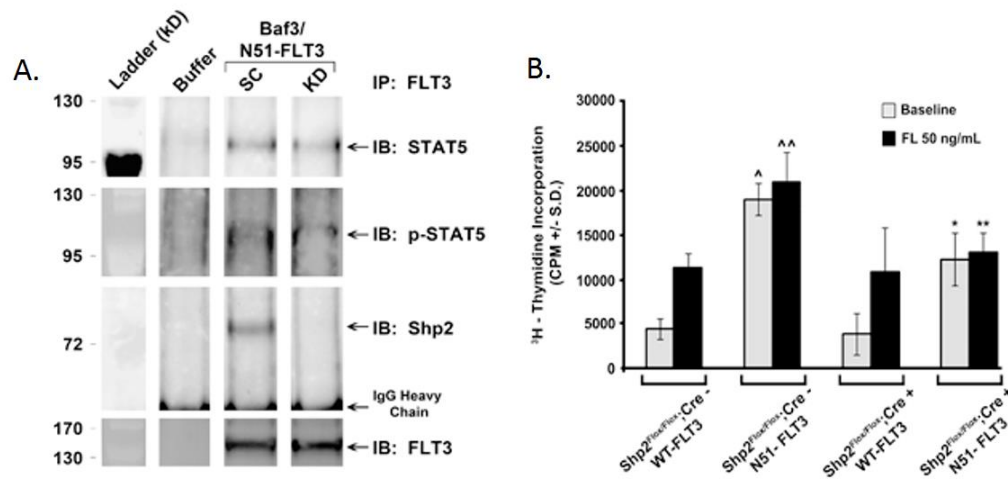


Figure 1.5: Genetic knock-down of Shp2 does not ablate STAT5 interaction with FLT3-ITD, and genetic disruption of Shp2 does not completely normalize N51-FLT3-induced proliferation.

- (A) Total cellular proteins from Baf3/N51-FLT3 cells transfected with scrambled shRNA (SC) or Shp2-specific shRNA (KD) were immunoprecipitated (IP) with anti-FLT3 and immunoblotted (IB) with anti-STAT5, anti-phospho-STAT5, anti-Shp2, and anti-FLT3.
- (B) ^3H -thymidine incorporation assay of transduced, sorted bone marrow low density mononuclear cells from Shp2^{flx/flx};Mx1Cre⁻ and Shp2^{flx/flx};Mx1Cre⁺ mice in the absence and presence of FL 50 ng/mL; two independent experiments combined with n=4 replicates per experiment, $^{\wedge}p<0.0001$ for N51-FLT3 v. WT-FLT at baseline in Cre⁻ cells, $^{\wedge\wedge}p<0.0001$ for N51-FLT3 v. WT-FLT in response to FL in Cre⁻ cells, $^*p<0.0001$ for N51-FLT3 in Cre⁺ cells v. Cre⁻ cells at baseline, and $^{**}p<0.0001$ for N51-FLT3 in Cre⁺ cells v. Cre⁻ cells in response to FL, statistical analysis performed using random effects ANOVA. Adapted from Nabinger, et al. (2013).

PI3K in AML

PI3K comes from a family of lipid kinases that generates second messengers which promote proliferation, cellular survival, and migration. Cancer genetics studies suggest that the PI3K pathway is the most frequently altered pathway in human tumors (Samuels, 2006). This strong genetic evidence has led to considerable interest and effort into the development of small molecule inhibitors targeting the PI3K–Akt–mTOR signaling pathway (Fruman, 2014). Although there have been encouraging results using pharmacologic PI3K inhibition in chronic lymphoid leukemia (CLL), the mechanism and rationale for targeting PI3K in AML has been understudied (Hoellenriegel, 2011).

Although there is a dearth of studies exploring PI3K inhibition in FLT3-ITD positive AML, there is some prior work suggesting a cooperative relationship between Shp2, PI3K, and WT-FLT3. Previous investigators have defined that Y768, Y955, and Y969 on WT FLT3 recruit the adapter proteins Grb2, Gab2, and the regulatory subunit of PI3K, p85 α , and that mutation of all of these tyrosine (Y) sites to phenylalanine (F) results in reduced phospho-Akt levels (Masson, 2009). As shown in figure 1.6, prior work in our lab has also suggested a relationship with FLT3-ITDs, as we saw reduced p-Akt levels following administration of II-B08, a Shp2 phosphatase inhibitor (Zhang, 2010). Given that Shp2 is reported to participate in multi-protein complexes containing Gab2 and p85 α in WT FLT3-expressing cells in response to FL stimulation (Zhang, 1999; Zhang, 2000), we hypothesized that Shp2 may cooperate with PI3K by associating with Gab2-containing complexes at FLT3-ITD Y768, Y955, and/or Y969.

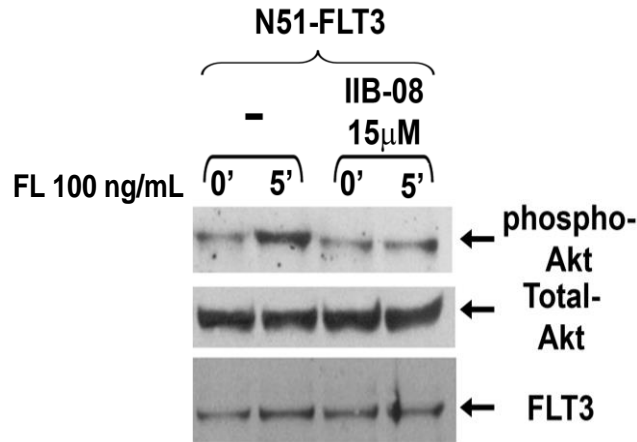


Figure 1.6: Inhibition of Shp2 decreases levels of p-Akt in N51-FLT3 cells.

Immunoblot demonstrating that pharmacologic inhibition of Shp2 with II-B08 leads to decreased levels of p-Akt in cells expressing N51-FLT3.

Summary

AML is a heterogenous disease that has cytogenetic and molecular signatures bearing prognostic significance. One important prognostic marker is the presence or absence of FLT3-ITD mutations, which have been shown to confer a poor prognosis. In particular, the molecular mechanisms behind FLT3-ITD-induced leukemia are not yet fully elucidated. Prior work in the Chan lab has shown a positive role for Shp2 in FLT3-ITD-induced hyperproliferation and STAT5 activation as well as an increase in survival of mice with genetic knockout of Shp2 (Nabinger, 2013). However, the cellular hyperproliferation and aberrant expression of p-STAT5 was not fully normalized following genetic inhibition of Shp2, suggesting alternate or additional pathways promoting FLT3-ITD-induced leukemia. Prior work has implicated a signaling relationship between PI3K, Shp2, and WT-FLT3 (Masson, 2009; Zhang, 1999; Zhang, 2000), and our lab has found a reduction of p-Akt in N51-FLT3-expressing cells upon pharmacologic inhibition of Shp2. Taken together, these findings suggest a potential cooperative relationship between Shp2 and PI3K in FLT3-ITD positive AML. Therefore, our current project explored two aims:

Aim 1. We hypothesize that mutation of Y768, Y955, or Y969 will result in reduced FLT3-ITD-induced hyperproliferation in vitro and reduced myeloproliferative disease in vivo due to reduced PI3K-Akt pathway activation.

Aim 2. We hypothesize that dual pharmacologic inhibition of both Shp2 phosphatase activity and PI3K activity will work cooperatively to inhibit FLT3-ITD-induced hyperproliferation in vitro.

To address the first aim, Y768, Y955, and Y969 were mutated to phenylalanine (F), alone and in combination, and constructs were introduced into 32D cells to determine which tyrosine residue or combination of tyrosine residues are most important for FLT3-ITD-induced hyperproliferation. To address the second aim, we tested the anti-proliferative effect of the Shp2 inhibitor, II-B08, and PI3K inhibitor, GDC-0941, in FLT3-ITD expressing 32D cells and in primary AML patient samples.

Significance

Given that the majority of AML patients are of advanced age, and cannot tolerate the harsh chemotherapy regimens used for standard treatment, they often are relegated to supportive care. Therefore, there is an urgent need to develop less toxic therapies to address this growing disease population. Prior work in the Chan lab has elucidated a positive role for Shp2 in FLT3-ITD induced malignant disease. However, the leukemic phenotype was not completely normalized following Shp2 inhibition alone, which led to our hypothesis that there are additional signaling molecules or pathways contributing to disease progression. Our current study implicating PI3K in FLT3-ITD induced AML is significant because it will be the first study to provide a rationale for PI3K inhibition specifically in FLT3-ITD positive AML. Furthermore, given the commercial development and access to PI3K inhibitors, the feasibility of moving to clinical trials makes this an area of study particularly amenable to translation from the bench to the bedside.

CHAPTER TWO

MATERIALS AND METHODS

A. Materials

1. Plasmids

pMSCV

pMSCV: WT-, N51-, and N51-point mutant constructs were expressed using the Murine Stem Cell Virus (pMSCV) plasmid. cDNA from human WT-FLT3 or patient derived FLT-ITD, N51-FLT3, were sub-cloned in the pMSCV vector co-expressing green fluorescent protein (EGFP) as a marker. N51-point mutations were generated with site-directed mutagenesis using QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to yield amino acid changes Y768F, Y955F, Y969F, Y768F/Y955F, Y768F/Y969F, Y955F/Y969F, and Y768F/Y955F/Y969F. After sequencing the cDNAs to rule out unwanted mutations the flag-tagged FLT3 point mutants were isolated using BamH1 digestion followed by gel electrophoresis and extraction. The plasmid pMSCV was then cut using Bgl II digestion, followed by a phosphatase reaction to prevent the vector from ligating back together. To move the FLT3 insert into pMSCV plasmid, a molar ratio of 1:3 of vector to insert was used, along with DNA ligase. After bacterial transformation, colonies were picked and expanded, plasmid DNA was extracted, and an Xho1 restriction enzyme digest was performed followed by gel electrophoresis. The Xho1 digestion was able to confirm directionality of the sub-cloned insert since the FLAG-FLT3 cDNA fragment had BamH1 sites on both ends and could be ligated into the BglII-digested MSCV plasmid in both orientations. Xho1 was chosen since it has an eccentrically located restriction site within the FLAG-FLT3 insert, as well as a site on the vector, allowing directionality to be confirmed based upon fragment size. If the orientation was correct, bands at 1624 bp and 552 bp would be present, whereas the alternative orientation would have bands at 1624 bp and 924 bp. Once correct directionality was confirmed, constructs were transfected into nascent 32D cells. Transfection was performed using the Amaxa™ cell line Nucleofector™ kit V, with the provided protocol.

Table 2.1: Oligonucleotide primers used for site-directed mutagenesis.

Primer		Primer Sequence
Y768F	Forward	CTCTGAAGATGAAATTGAATTTGAAAACCAAAAAAGGCTG
	Reverse	CAGCCTTTTTTGGTTTTCAAATTCAATTTTCATCTTCAGAG
Y955F	Forward	CAGAAGAAGCGATGTTTCAGAATGTGGATGG
	Reverse	CCATCCACATTCTGAAACATCGCTTCTTCTG
Y969F	Forward	GAATGTCCTCAGACCTTCCAAAACAGGCGACC
	Reverse	GGTCGCCTGTTTTGGAAGGTCTGAGGACATTC

2. Patient Samples

Patient peripheral blood or bone marrow aspirate samples were obtained at the time of diagnostic testing and with prior patient consent. Approval was obtained from Institutional Review Boards of the Indiana University School of Medicine. Using Ficoll-Hypaque, low density mononuclear cells were isolated for in vitro proliferation assays.

3. Antibodies

Table 2.2: Primary antibodies

Primary antibodies	Company	Clone
T-Akt	Cell Signaling	N/A
P-Akt (S473)	Cell Signaling	587F11
P-Erk	Cell Signaling	N/A
T-Erk	Cell Signaling	N/A
P-Stat5 (Y694)	Cell Signaling	N/A
T-Stat5	Cell Signaling	3H7
T-FLT3	Santa Cruz	C-20
P-FLT3 (Y591)	Cell Signaling	N/A
GAPDH	Biodesign International	6C5

Table 2.3: Secondary antibodies

Secondary antibodies	Company
Goat Anti-Rabbit IgG HRP	Santa Cruz
Goat Anti-Mouse IgG HRP	Santa Cruz

4. Kits

Table 2.4: Kits

Kit	Company	Catalog Number
Plasmid Maxi-Prep Kit	Invitrogen	K459501
Mini-prep Kit	Qiagen	27106
Nucleofector Kit V	Amaza/Lonza	VCA-1003
QIA quick gel extraction kit	Qiagen	28704

5. Mice

C3H/HeJ

C3H/HeJ mice were received from Harlan and used for transplant studies.

B. Methods

1. Cell culture

32D cells

32D cells, American Type Culture Collection (ATCC) were cultured in IMDM (Invitrogen), 10% FBS (HyClone), 1% Penicillin/streptomycin (Invitrogen), and 10 ng/ml murine IL-3 (PeproTech). Cells were grown in a humidified incubator at 37°C with 5% CO₂. Cells were passaged approximately every 2-4 days.

2. Transfection

32D cells

Approximately 1.0X10⁶ 32D cells were mixed with 1 µg each of pMSCV-FLT3 construct, followed by exposure to an electric current delivered via a Nucleofector 2b device®.

3. Cell Sorting

Transfected cells were sorted using fluorescence-activated cell sorting (FACS) and enhanced green fluorescent protein-expressing (EGFP+) cells were cultured for functional and biochemical studies.

4. Thymidine Incorporation Assays

Cells were starved for 4-6 hours in serum-free IMDM and then plated into thymidine incorporation assays either at baseline (no growth factor) or in the presence of 100ng/ml FLT3 ligand (FL), with or without inhibitor, and incubated overnight at 37°C. The next morning, cells were pulsed with 1µCi tritiated thymidine (³H) and incubated for 4-6 hours at 37°C. ³H-Thymidine incorporation was determined using an automated 96-well cell harvester (Brandel, Gaithersburg, MD).

5. Western blots

Sorted, EGFP⁺ cells were collected and protein extracts were prepared as previously described (Shi, 2000). Protein extracts were quantified using Bradford reagent (Bio-Rad, Hercules, CA) against protein standards, and read using a Spectra-Max® 340 pc microplate reader. Extracts were then electrophoresed and transferred to nitrocellulose as previously described (Chan, 2005). All signals were detected by enhanced chemiluminescence and p-STAT5 and p-Erk signals were quantitated using ImageJ software (National Institutes of Health, Bethesda, MD).

6. Transplants

2.0×10^6 32D cells expressing each of the various constructs including: WT-FLT3, N51-FLT3, and N51-FLT3-Y768F were injected via tail vein into C3H/HeJ mice by the In Vivo Therapeutics Core at the Indiana University School of Medicine. Transplanted animals were followed for overall survival, and results were analyzed using Kaplan Meier method, significance at 0.05 level.

7. Statistical Analysis

For combined data from modified N51-FLT3 constructs, unpaired, two-tailed, Student's *t* test was used. For Western blot quantification using modified N51-FLT3 constructs, paired or unpaired, two-tailed, Student's *t* test was used. For the transplant study, overall survival was analyzed using Kaplan Meier estimation where death was the pertinent event. P values were generated using the log-rank tests. For effect of II-B08 and GDC-0941 on N51-FLT3 cell and primary human AML cell proliferation, unpaired, two-tailed, Student's *t* test was used. For all analyses, statistical significance was set at 0.05.

RESULTS

Generation of FLT3-ITD constructs and FLT3-ITD-expressing 32D cells

The original N51-FLT3-ITD cDNA was subjected to site-directed mutagenesis to generate various tyrosine (Y) to phenylalanine (F) mutants (Figure 2.1). Nascent 32D cells were transfected with MSCV-WT-FLT3, N51-FLT3, and the various N51-FLT3 tyrosine (Y) to phenylalanine (F) constructs. Afterwards, the cells were sorted by FACS and enriched for EGFP positivity, until there were sufficient numbers of cells to perform assays. As an initial pilot experiment, the transfected cells were evaluated for proliferation using ³H-thymidine incorporation assay, as shown in Figure 2.2. We consistently saw reduced proliferation with the single mutant N51-FLT3-Y768F with and without FL. However, for the other single mutants, we observed mixed results including only a modest effect of the N51-FLT3-Y955F mutant and paradoxically increased proliferation of cells expressing N51-FLT3-Y969F when cultured in IL3 (data not shown). Collectively, these pilot studies suggested that the Y955 or Y969 sites may actually function to recruit inhibitory signaling proteins rather than to recruit activating, stimulatory signaling proteins. Based upon the established role of Y768 and the reproducibility of proliferation reduction observed in the pilot studies, we focused on the role of Y768 in N51-FLT3-ITD-induced biochemical signaling and development of myeloproliferative neoplasm in vivo.

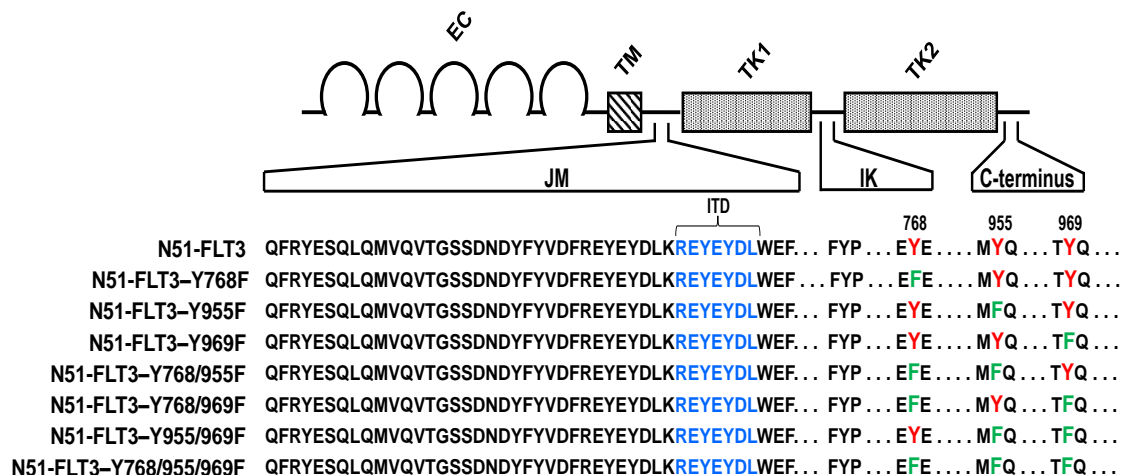


Figure 2.1: Schematic diagram showing N51-FLT3 constructs.

Schematic representation of tyrosine residues that were mutated from tyrosine (Y) to phenylalanine (F) alone and in combination. EC – extracellular, TM- transmembrane, JM-juxtamembrane, TK-1 – kinase domain, TK-2 – kinase domain

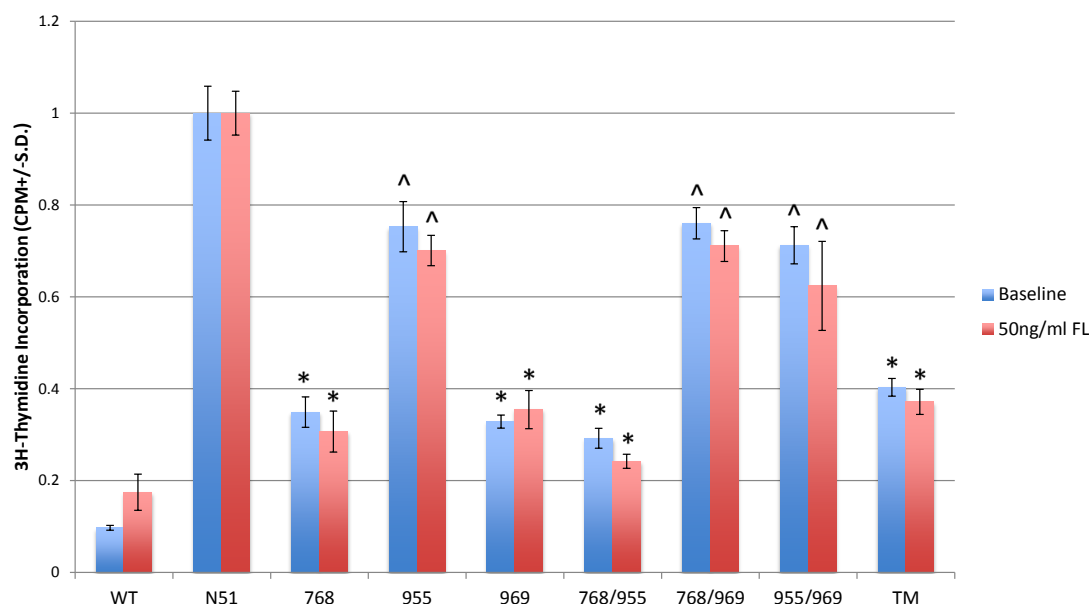


Figure 2.2: ^3H -thymidine incorporation experiment, using all modified N51-FLT3 constructs.

Murine 32D cells were transfected with WT-FLT3, N51-FLT3, N51-FLT3-Y768F, N51-FLT3-Y955F, N51-FLT3-Y969F, or combinations, sorted by FACS, and subjected to ^3H -thymidine incorporation assay in the absence and presence of FL 50 ng/mL; representative of three independent experiments with similar results, n=4 replicates per experiment, data normalized to N51-FLT3 proliferation levels.

*p<0.0001 for 768, 969, 768/955, or TM v. N51-FLT3 at baseline and in the presence of FL 50 ng/mL, ^p<0.05 for 955, 768/969, or 955/969 v. N51-FLT3 at baseline and in the presence of FL 50 ng/mL, statistical analysis using unpaired, two-tailed, student's *t* test.

Mutation of N51-FLT3 tyrosine (Y) 768 to phenylalanine (F) diminishes FLT3-ITD-induced hyperproliferation

Given the promising results we obtained using the N51-FLT3-Y768F mutant in reducing proliferation in pilot studies, we focused the remainder of our work on that mutant. In carrying these studies forward, we found a sequence error in the original N51-FLT3-ITD-Y768F construct; therefore, the construct was re-generated, re-sequenced, and re-evaluated for proliferation (Fig. 2.3). Again, we once again

saw reduced proliferation with mutation of tyrosine (Y) 768 to phenylalanine (F) (Figure 2.3); however, the levels were not corrected to WT levels, suggesting involvement of multiple signaling pathways. As previous investigators had found that the point mutants WT-FLT3-Y768F, WT-FLT3-Y955F, and WT-FLT3-Y969F were relevant for PI3K signaling, we wanted to investigate if the N51-FLT3-Y768F mutant caused reduced activation of Akt, Erk, and STAT5 (Masson, 2009).

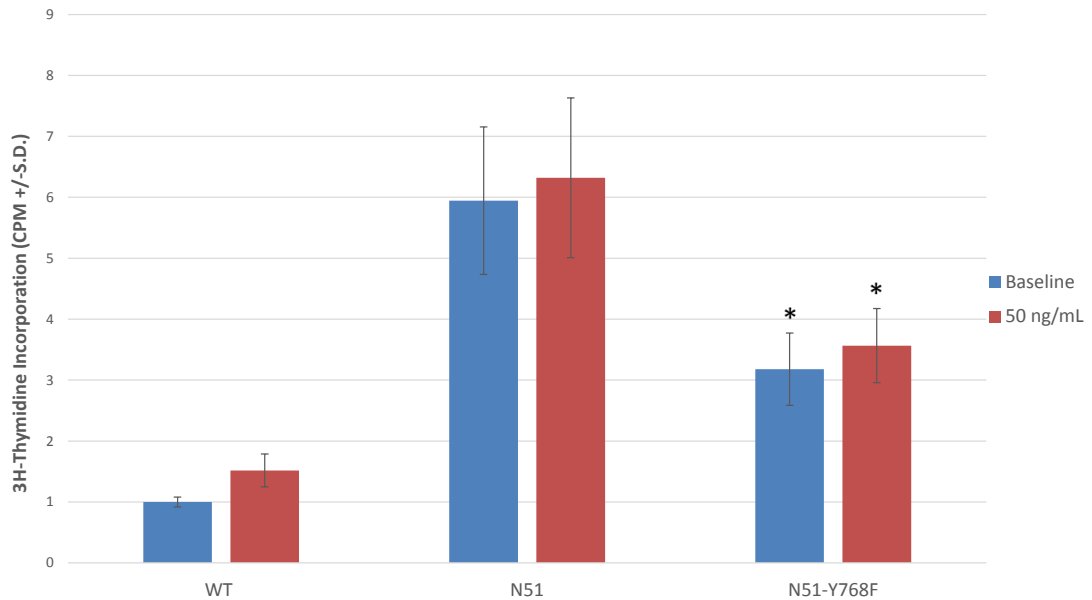


Figure 2.3: Mutation of N51-FLT3 tyrosine (Y) 768 to phenylalanine (F) diminishes FLT3-ITD-induced hyperproliferation.

Murine 32D cells were transfected with WT-FLT3, N51-FLT3, or N51-FLT3-Y768F, sorted by FACS, and subjected to ³H-thymidine incorporation assay in the absence and presence of FL 50 ng/mL. Proliferation was normalized to average WT FLT3-expressing cells at baseline within each independent experiment, data compiled from four independent experiments, *n=4, p<0.05 comparing N51-FLT3-Y768F to N51-FLT3 in the absence or presence of FL, statistics performed by unpaired, two-tailed student's t-test.

Mutation of N51-FLT3 tyrosine (Y) 768 to phenylalanine (F) normalizes Erk , but not Akt or STAT5 activation

As we observed reduced cellular proliferation with N51-FLT3-Y768F, we next examined its functional role with biochemical analysis. In cells expressing N51-FLT3-Y768F, there were reduced levels of phospho-Erk compared to N51-FLT3 expressing cells, with no observed effect on levels of phospho-STAT5 (Figure 2.4). Also of note, we saw no difference in phospho-Akt levels between WT-FLT3 and N51-FLT3 expressing cells, and mutation of Y768 did not reduce Akt activation compared to N51-FLT3. Together, these findings implicate Y768 as being relevant for positive regulation of the RAS-Erk signaling pathway in FLT3-ITD positive AML, but having minimal or no effect on PI3K-Akt or STAT5 activation.

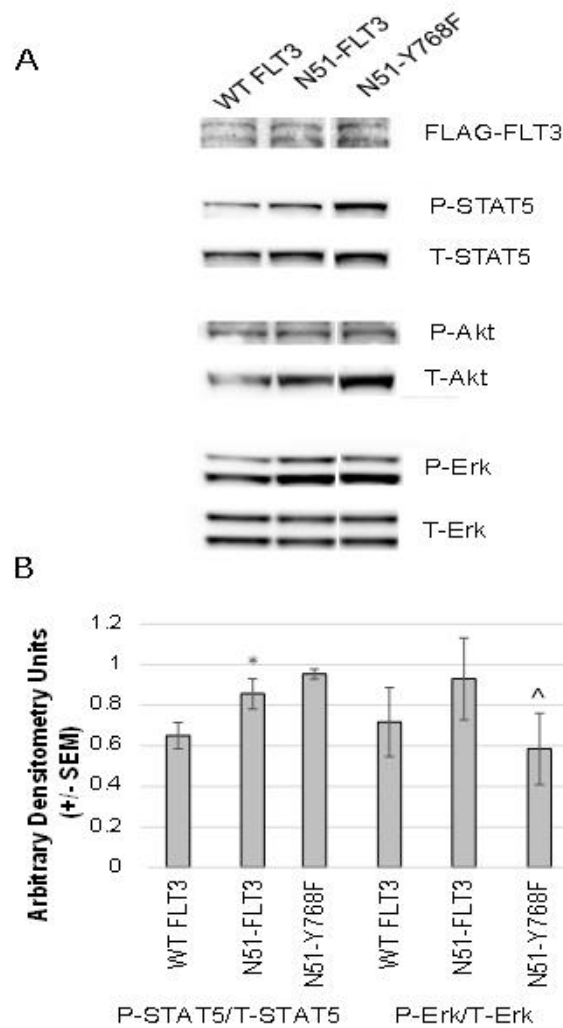


Figure 2.4: Mutation of N51-FLT3 tyrosine (Y) 768 to phenylalanine (F) normalizes Erk , but not Akt or STAT5 activation

(A) Immunoblot analysis of STAT5 phosphorylation, total STAT5, Akt phosphorylation, total Akt, Erk phosphorylation, total Erk, and FLAG-FLT3 expression in transfected and sorted 32D cells expressing WT-FLT3-, N51-FLT3-, or N51-FLT3-Y768F, repeated in 3 – 5 independent experiments. (B) Band intensities were quantitated using densitometry. Data were compiled from the experiments and demonstrated graphically. Data shown as \pm SEM; * $n=3$, $p<0.05$ comparing p-STAT5/t-STAT5 in N51-FLT3- to WT FLT3-expressing cells (paired, two-tailed student's t test) and ^ $n=5$, $p<0.01$ comparing p-Erk/t-Erk in N51-FLT3-Y768F- to N51-FLT3-expressing cells (unpaired, two-tailed student's t test).

Mutation of N51-FLT3 tyrosine (Y) 768 to phenylalanine (F) has no effect on Myeloproliferative disease (MPD) in vivo

Given the reduced cellular proliferation and reduced Erk activation observed in vitro, the next step was to determine if mutation of Y768 would alter overall survival in an in vivo model. A syngeneic transplant using C3H/HeJ mice (Figure 2.5) was performed, and groups were followed for 70 days. We observed no difference in survival between N51-FLT3 mice and N51-FLT3-Y768F mice (Figure 2.6) suggesting that mutation of this residue alone is not sufficient to affect the progression of myeloproliferative disease in vivo. One possibility is that, in a physiologic model, preserved Akt and STAT5 signaling pathways may be sufficient to overcome the reduced cellular proliferation and Erk signaling seen during in vitro experiments.

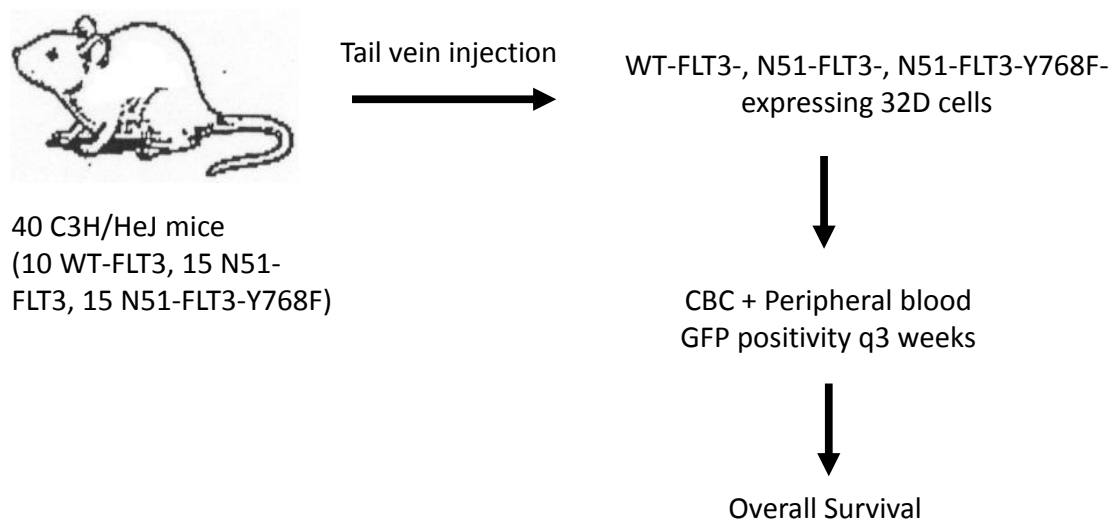


Figure 2.5: Transplant protocol for N51-FLT3 construct survival study.

Schematic diagram showing transplant design. C3H/HeJ mice were injected by the In Vivo Therapeutics Core at IUSM with 3.0×10^6 32D cells expressing WT-FLT3, N51-FLT3, or N51-FLT3-Y768F and followed for overall survival.

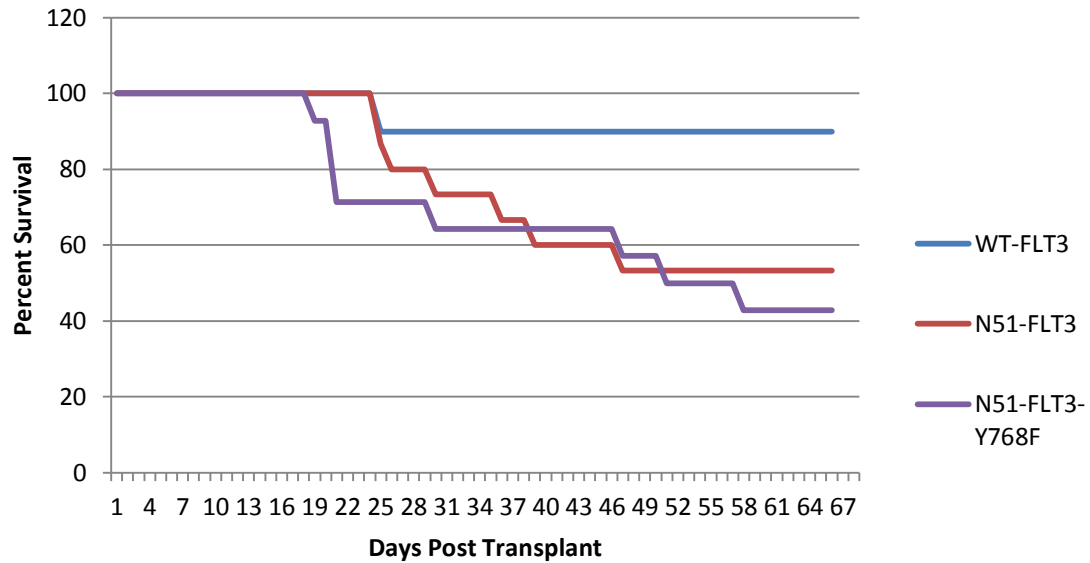


Figure 2.6: Mutation of N51-FLT3 tyrosine (Y) 768 to phenylalanine (F) has no effect on Myeloproliferative disease (MPD) in vivo.

Kaplan-Meier analysis of overall survival for all mice transplanted with 32D cells expressing WT-FLT3, N51-FLT3, or N51-FLT3-Y768F, n=10 in the WT-FLT3 group and n=15 in the N51-FLT3 and N51-FLT3-Y768F groups respectively; N51-FLT3 and N51-FLT3-Y768F groups not significantly different.

Pharmacologic inhibition of Shp2 and PI3K reduces FLT3-ITD induced cellular hyperproliferation

Since prior work has implicated a signaling relationship between PI3K, Shp2, and WT-FLT3 (Masson, 2009; Zhang, 1999; Zhang, 2000), and our lab has found a reduction of p-Akt in N51-FLT3-expressing cells upon pharmacologic inhibition of Shp2 (Figure 1.6), we wanted to investigate if there was a cooperative relationship between Shp2 and PI3K in FLT3-ITD positive AML. We utilized the Shp2 inhibitor, II-B08 (IC₅₀ of 5.5 μ M), which has been shown to be critical for the activation of Erk (Zhang, 2010), and the PI3K inhibitor, GDC-0941 (IC₅₀ of 3 nM), as PI3K is the upstream activator of Akt (Folkes, 2008). The Shp2 inhibitor, II-B08, has been used previously and published by us (Zhang, 2010). The PI3K inhibitor, GDC-0941, is a commercially available compound currently in phase 2 clinical trials for non-small cell cancer (NSCLC) (Genentech; ClinicalTrials.gov, Identifier-NCT01493843). Using WT-FLT3 and N51-FLT3-expressing 32D cells, we observed that the N51-FLT3-expressing cells showed a significant reduction of proliferation with II-B08 or GDC-0941 alone (Figure 2.7A). We similarly found reduction in the proliferation of primary AML samples upon administration of II-B08 or GDC-0941 alone (Figure 2.7B). While both II-B08 and GDC-0941 independently demonstrated an effect on proliferation, we did not see a strong cooperation of this drug combination in the FLT3-ITD-expressing 32D cells or in the primary AML patient samples examined (n=4). However, after examining increased numbers of patient samples (Briana Richine, Chan Lab), we did find that the addition of IIB-08 to GDC-0941 significantly reduced primary AML cell proliferation compared to treatment with GDC-0941 alone (data not shown).

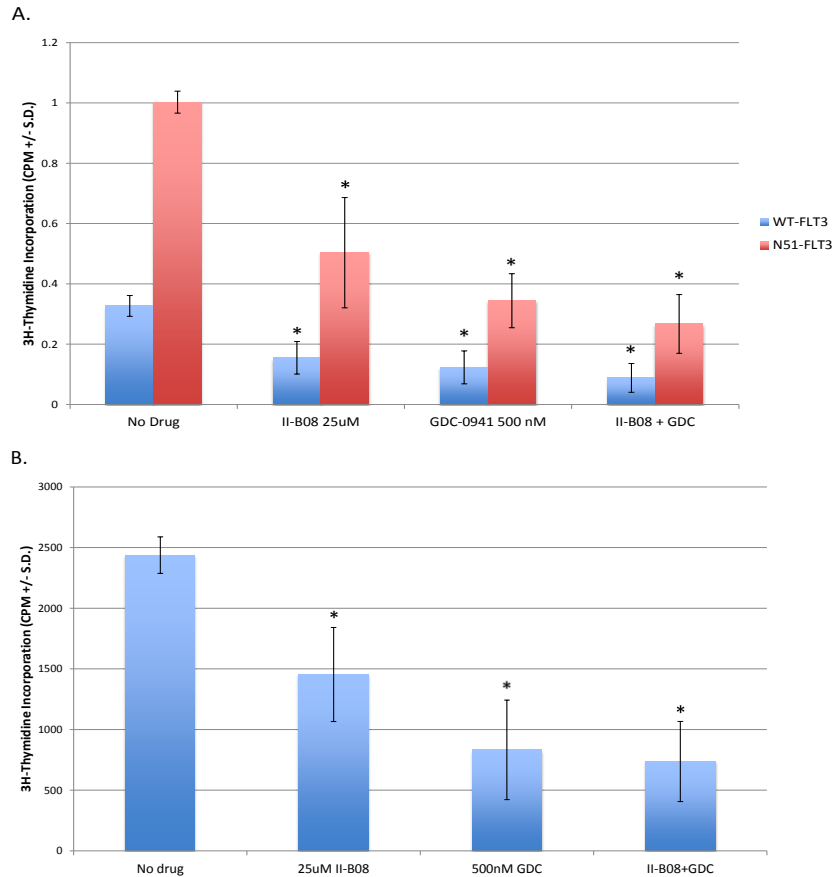


Figure 2.7: Pharmacologic inhibition of Shp2 and PI3K reduces FLT3-ITD induced cellular hyperproliferation

(A) ^3H -thymidine incorporation assay of 32D cells transfected with either WT-FLT3 or N51-FLT3 in the absence or presence of II-B08 and/or GDC-0941; three independent experiments combined with n=3-4 replicates per experiment, data normalized to N51-FLT3 proliferation levels, * $p < 0.0001$ for no drug (N51-FLT3 or WT-FLT3) vs. II-B08, GDC-0941, or II-B08 + GDC-0941 for WT or N51 respectively, statistical analysis performed using two-tailed, unpaired, student's t-test.

(B) ^3H -thymidine incorporation of primary AML cells cultured in GM-CSF 1 ng/mL + FL 50 ng/mL, data normalized to proliferation levels in the absence of drug for each sample, n=4 patient samples total, * $p < 0.0001$ for no drug v. II-B08, GDC-0941, or II-B08 + GDC-0941, statistical analysis performed using two-tailed, unpaired, student's t-test.

CHAPTER THREE

DISCUSSION

AML is a lethal disease with a poor overall prognosis, highlighting the need to better understand the mechanisms behind disease progression and bring forth potential novel therapeutic targets. Given that FLT3-ITDs are known markers for poor prognosis in AML, cultivating a better understanding of their signaling pathways may lead to insights that can be applied to the overall disease process. In this study, we investigated the role of Y768 in vitro and in vivo with 32D cells expressing N51-FLT3, as well as the effect of pharmacologic inhibition of Shp2 and PI3K in vitro.

Beginning with 32D cells transfected with WT-FLT3, N51-FLT3, or N51-FLT3-Y768F, we first observed that the Y768 residue is important for cellular proliferation as well as Erk activation. While Masson et al. found that Baf3 cells transfected with ITDs containing triple mutation of Y768, Y955, and Y969 had reduced cell survival and decreased MAPK and PI3K signaling, our studies further define that Y768 alone appears to be a relevant residue for the positive regulation of Erk activation in FLT3-ITD positive AML. Additionally, in our experiments we observed no decrease in STAT5 activation upon mutation of the Y768 residue. One possibility for the lack of difference observed in STAT5 phosphorylation between N51-FLT3 and N51-FLT3-Y768F might be compensation from other relevant tyrosine residues – specifically Y955 and Y969. Another possibility could be contributions from the non-receptor tyrosine kinase, spleen tyrosine kinase (SYK), which has been shown to contribute towards STAT5 signaling at other sites along the FLT3-ITD receptor (Puissant, 2014; Kanie, 2004). The lack of observed effect on Akt activation may be related to alternative pathways in the 32D cell line, given that WT-FLT3 and N51-FLT3 were not significantly different.

Next, we explored the importance of Y768 in myeloproliferative disease (MPD) in an in vivo model. As expected, animals transplanted with N51-FLT3-expressing cells had decreased survival; however, when comparing N51-FLT3 to N51-FLT3-Y768F we saw no improvement in overall survival (Figure 2.6). The decreased survival observed with N51-FLT3-Y768F is inconsistent with the findings

of Puissant et al., who found that mice transplanted with constitutively activated SYK (SYK-TEL oncogene) and FLT3-ITD-Y768A or FLT3-ITD-Y768/955AA mutant construct did not succumb to disease. Our study did not use the SYK-TEL oncogene in animal studies, and these differing results may indicate that oncogenes such as SYK may be playing a role in the disease process. Furthermore, it is worth noting that we did not see any effect on STAT5 activation with our N51-FLT3-Y768F-expressing cells in vitro. Given that promiscuous activation of STAT5 is one of the hallmarks of FLT3-ITD positive AML, it is unlikely that correcting levels of p-Erk alone would be sufficient to modify the disease phenotype in vivo. Other members of the lab are actively investigating the role of SYK in N51-FLT3-ITD, which may provide insight into the results observed during our current study.

Our lab has previously established Shp2 as a potential therapeutic target in FLT3-ITD positive AML. Given the mounting evidence of PI3K involvement in FLT3-ITDs (Masson, 2009; Han, 2015), we further extended our study to include inhibition of PI3K in addition to Shp2 in an in vitro model. We found that pharmacologic inhibition of Shp2 with II-B08 led to decreased proliferation in N51-FLT3-expressing 32D cells as well as primary AML samples, as did administration of the PI3K inhibitor GDC-0941 (Figure 2.7). However, although there was a trend towards a cooperative effect observed between administration of both II-B08 and GDC-0941, it was not statistically significant (although further data collected in the lab has shown cooperativity in primary AML samples).

Overall, our biochemical and pharmacologic findings confirm the importance of Shp2 in FLT3-ITD-induced leukemogenesis and implicate involvement of PI3K. We observed consistent findings with the N51-FLT3-Y768F mutant and reduction of Erk signaling. We also show that pharmacological inhibition of Shp2 or PI3K can decrease cellular hyperproliferation induced by FLT3-ITDs. Altogether, this study supports the involvement of PI3K and Shp2 in AML, and suggests that pharmacologic inhibition of Shp2 and/or PI3K may provide a novel approach for treatment.

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CURRICULUM VITAE

Joshua D. Bowling

EDUCATION

Indiana University May 2016 *Indianapolis, IN*
M.D.

Indiana University July 2016 *Indianapolis, IN*
M.S. in Translational Science

Manchester University May 2011 *North Manchester, IN*
B.A., Biology/Chemistry, *magna cum laude*

GRANTS & AWARDS

2012 Poster Presentation Award, Student Research in Academic Medicine
(SRPinAM) poster presentation, Indiana University School of Medicine

2013 Indiana CTSI MD/MS Fellowship

RESEARCH EXPERIENCE/PRESENTATIONS

May, 2012 – August 2012, Student Research in Academic Medicine (SRPinAM)
Rebecca J. Chan, M.D., Ph.D., Faculty Mentor
Department of Pediatrics, Indiana University School of Medicine

Project: “Role of the Protein Tyrosine Phosphatase, Shp2, in FLT3-ITD+ Leukemia”

- Oral Presentation: August 10, 2012
- Poster Presentation: October 4, 2012 (received poster presentation award)

June, 2013 – May, 2014, Indiana CTSI MD-MS Program
Rebecca J. Chan, M.D., Ph.D., Faculty Mentor

Thesis Committee: Sophie Paczesney, M.D., Ph.D. and Michael Ferguson, M.D.
Department of Pediatrics, Indiana University School of Medicine

Project: "Shp2 and PI3K Inhibition in FLT3-ITD-Induced Leukemia"

- Oral Presentation: March 28, 2016

MANUSCRIPTS

1. Richine BM, Virts EL, **Bowling JD**, Ramdas B, Mali R, Naoye R, Liu Z, Zhang Z-Y, Boswell HS, Kapur R, and Chan RJ. 2016. Syk kinase and Shp2 phosphatase inhibition cooperate to reduce FLT3-ITD-induced STAT5 activation and proliferation of acute myeloid leukemia. *Leukemia* [in press].

2. Nabinger SC, Li XJ, Ramdas B, He Y, Zhang X, Zeng L, Richine BM, **Bowling JD**, Fukuda S, Goenka S, Liu Z, Feng GS, Yu M, Sandusky GE, Boswell HS, Zhang ZY, Kapur R, Chan RJ. 2013. The protein tyrosine phosphatase, Shp2, positively contributes to FLT3-ITD-induced hematopoietic progenitor hyperproliferation and malignant disease in vivo. *Leukemia* Feb;27(2):398-408.

ABSTRACTS

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